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Article

Separation and Evaluation of Soybean Protein Hydrolysates Prepared by Immobilized Metal Ion Affinity Chromatography with Different Metal Ions

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Metal ion affinity chromatography is widely used to purify peptides on the basis of the dissimilarities of their amino acids. However, researchers are interested in the separation differences between different metal ions in this method. In our study, four kinds of commonly used metal ions are compared by the amount of immobilized metal ion on iminodiacetic acid–Sepharose and binding amount of soybean peptide to immobilized iminodiacetic acid–Mn^{+} adsorbents and evaluated by high-performance liquid chromatography (HPLC) profiles. The results show that due to the different adsorption behaviors of metal ions, the binding ability order of soybean protein peptide on the column should be $Fe^{3+} > Cu^{2+} > Zn^{2+} > Ca^{2+}$. The HPLC profiles show that peptides adsorbed by four kinds of metal ions display similar strong hydrophobic characteristics.

Introduction

Soybean protein is an important food protein, enzymatic hydrolysis of which can release peptides that are able to chelate metal ions and show different biological activities such as antioxidation, lipid-lowering, gastrointestinal regulation, and absorption of trace elements (1–2). Recently, researchers from all over the world have paid attention to the fact that compounds like amino acids and peptides like casein phosphopeptides or egg yolk phosphopeptides can chelate calcium to form soluble complexes so that calcium does not deposit in the small intestine (3–4). Soybean protein is rich in glutaminic acid and aspartic acid and oxygen on carboxyl groups, which is able to chelate calcium ions. Kumagai reported that soybean globulins after phytate removal and deamidization have calcium-binding properties (5). We were interested in whether soybean protein hydrolysates possess such property, and therefore, examined how to prepare and purify soybean peptides according to their different functions and find the components playing a role.

Currently, the most commonly used peptide purification methods are gel chromatography, ultrafiltration and ion exchange chromatography. Gel chromatography possesses a chromatographic medium for which the separation is based on molecular size (6), and it is effective for peptide purification or separation in terms of molecular weight distribution. The most common applications of ultrafiltration are protein concentration (i.e., solvent removal), buffer exchange and desalting (7). Ion exchange chromatography offers resolution of impurities or product isoforms with differences in charge, and can be used to separate proteins or peptides with different charges. This method is also often used to remove endotoxin, nucleic acids and host cell proteins (8). However, these methods are not efficient for peptide purification by their differences in amino acids, especially those binding with metal ions.

Immobilized metal ion affinity chromatography (IMAC) was first proposed by Porath and was used to separate protein possessing amino acid residues, which are able to chelate metals (9). Amino acid residues such as His, Trp or Cys coordinate with metal ions such as Cu, Co, Zn, Fe or Al cations through nonbonding lone pair electron, while metal ions are chelated to a multidentate ligand and immobilized onto a support material. It is a versatile technique because the same metal ion can be used in the adsorption of different proteins and the same matrix can be used to chelate different metal ions. Because the dissimilarities of these amino acids in terms of type, amount, location and steric conformations, the affinities with metal complexes are different, and thus it is possible to selectively separate and purify the proteins.

However, it is still not clear that whether it is possible to obtain a large amount of metal ion-binding peptides by IMAC; the differences between peptides adsorbed by various metal ions are also unclear. In this article, four kinds of IMACs, that is, Cu^{2+}, Fe^{3+}, Zn^{2+} and Ca^{2+}, were employed to determine the optimal separation condition by comparing their metal ion chelating capacity, adsorption and separation efficiency of soy peptides.

Materials and Methods

Materials

The soybean protein isolates (SPIs) utilized in our study were self-made (protein content 85%) as described by Lv (10). Protease M (51.5 AU/g) and glutaminase (100 gtu/g) were purchased from Amano Enzyme Co. (Nagoya, Japan). Sepharose 6B was purchased from Pharmacia (Uppsala, Sweden). Iminodiacetic acid (IDA) was purchased from Sigma (St. Louis, MO). IDA–sepharose 6B (Vector Gene Technology Co.; Beijing, China) was prepared according to the method described by Porath (11). All other chemicals were of analytical reagent grade.

Peptide preparation

To prepare soybean protein hydrolysates (SPHs), the pH of the SPI solution was adjusted to 3.0 and then protease M was

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added (protein content of 20 g/L, E:S = 1:100). Enzymatic hydrolyses were then performed on these solutions at 50°C for 60 min and the solutions were denatured for 10 min in a boiling water bath (Huanfeng Co.; Jiang Su, China) following digestion. After cooling to room temperature, the solutions (percentage of soluble hydrolysates at approximately 38%) were centrifuged at 3,000 g for 20 min. Resulting supernatants (E:S = 1:50) were adjusted to pH 7.0 when glutaminase was added and hydrolyzed at 50°C for 180 min. After hydrolysis, the solutions were heated in a boiling water bath for 5 min to deactivate glutaminase, and then cooled to room temperature and centrifuged (Anting Instruments; Shanghai, China) (3,000 g, 20 min). The resultant supernatants were collected as SPHs. The deamidation rate of SPHs was approximately 6%. This solution was ultrafiltrated with membranes of molecular weight cutoffs (MWCO) of 10 and 30 kDa at 4°C. Finally, the ultrafiltration fractions between 10 and 30 kDa were lyophilized (12).

Preparation and regeneration of chromatography gels

Preparation

IDA–Sepharose 6B (15 mL) was loaded onto the column (2.6 × 10 cm) and then incubated overnight with 0.2 mol/L $FeCl_3$, $CaCl_2$, $ZnSO_4$ and $CuSO_4$ (five bed volumes), respectively, at 4°C for Fe^{3+}, Ca^{2+}, Zn^{2+} and Cu^{2+} to chelate with IDA. To remove the unbound metal ions, the gels were washed with distilled water for 3–5 bed volumes until the electrical conductivity of the washed out water was equal to the value of distilled water. Then the gels were washed with 0.05 mol/L sodium acetic acid (NaAC-HAC) buffer at pH 4.0 for 3–5 bed volumes. After this, they were equilibrated with NaAC-HAC buffer containing 0.1 mol/L NaCl at pH 5.5 for 3–5 bed volumes and NaAC-HAC buffer at pH 5.5 for 5–10 bed volumes. Thus, Fe^{3+}–IDA–Sepharose 6B, Ca^{2+}–IDA–Sepharose 6B, Zn^{2+}–IDA–Sepharose 6B and Cu^{2+}–IDA–Sepharose 6B were prepared.

Regeneration

The gels were first eluted by 0.05 mol/L EDTA for 5–10 bed volumes, followed with distilled water for 5–10 bed volumes, then 20% ethanol was used for 10 bed volumes, and then distilled water again for 10 bed volumes. The flow rate was 1 mL/min.

Peptide chromatographic procedures

The lyophilized ultrafiltration fraction (10 to 30 kDa) solution (20 mg/mL) was loaded onto the prepared columns and the unbond samples were washed with NaAC-HAC buffer containing 0.1 mol/L NaCl at pH 5.5. The absorbance of the eluate was monitored at 220 nm. The wash volume was collected until the absorption value was 0 and designated the nonadsorbed fraction. The bound peptides were afterwards eluted with the appropriate elution buffers and collected for further use [reverse phase (RP)-HPLC experiments]. The flow rate was 1 mL/min. All chromatographic experiments were carried out at room temperature.

Elution buffers

The elution buffers used in these experiments were as follows: (i) 0.02 mol/L Na_2HPO_4; (ii) 0.02 mol/L NaH_2PO_4; (iii) 0.02 mol/L NH_4Cl; (iv) 0.02 mol/L imidazole; (v) 0.02 mol/L sodium glutamate; (vi) 0.02 mol/L malonic acid.

Chelating capacity of metal ions on affinity chromatography gels

The columns were packed with prepared gels (15 mL) then eluted with 0.05 mol/L EDTA, so that Cu^{2+}, Fe^{3+}, Zn^{2+} and Ca^{2+} on the gels were sufficiently chelated with EDTA and washed off. The elutions containing Cu^{2+}, Fe^{3+}, Zn^{2+} and Ca^{2+} chelated with EDTA were collected to determine the metal ion contents by atomic absorption spectrometry.

Soybean peptide binding capacity of prepared affinity chromatography gels

The nonadsorbed fractions were collected following the procedures described previously. The peptide concentration of the nonadsorbed fractions was expressed by protein concentration, which was determined by the method of Lowry (13), with bovine serum albumin (BSA) as a reference protein. Thus, the unbound amount of peptides was determined. The binding capacity of affinity gels with different metal ions were calculated by the following formula:

Binding capacity (mg) = amount of samples loaded (mg) − amount of unbound peptides (mg).

RP-HPLC

The previously obtained adsorbed peptides were lyophilized and dissolved in the initial solvent [water with 0.1% (v/v) trifluoroacetic acid (TFA)]. The solutions were applied to RP-HPLC on a protein and peptide Zorbax SB-C18 column (4.6 × 250 mm, 5 μm) from Agilent Technologies (Miford, MA). The column was equilibrated using water with 0.1% (v/v) TFA at 1 mL/min flow rate. The linear gradient of methanol was applied from 0 to 100%. Elution was monitored at 214 nm.

Metal content determination

Ca, Fe, Zn and Cu contents were determined by air-acetylene flame atomic absorption spectrometry (FAAS), which was from PuXiTongYong Co. (Beijing, China).

Cu, Zn, Fe and Ca single-element standard stock solutions (State Center for Standard Matter) were serially diluted by 3% nitric acid solution similar to the standard working solutions. The standard solutions (concentrations from low to high) were

Table I
Operating Parameters of Air-Acetylene Flame Atomic Absorption Spectrometry

Element	Current (mA)	Wavelength (nm)	Slit (nm)	Air flow (min/L)	Acetylene gas flow (min/L)
Cu	9.0	324.8	1.3	15.0	2.2
Zn	6.5	213.9	1.3	15.0	2.0
Fe	15.0	248.3	0.2	15.0	2.0
Ca	9.0	422.7	2.4	15.0	2.4

determined by FAAS according to the operating parameters in Table I. The FAAS instrument automatically processes the data and displays the standard curve, after which it measures the blank and sample solutions (diluted to the linear range) by sequence. Then the computer automatically displays the measurement results; the read value is the concentration of the sample.

Statistical analysis

The values are reported as the means ± standard deviation (SD), $n = 2$. Analysis of variance (ANOVA) was used to determine the significance of the differences of the data: $P < 0.05$ was considered to be significant. Statistical analysis was performed using the OriginPro 7.5 software package (OriginLab Corp.; Northampton, MA).

Results and Discussion

Comparison of the capacity of affinity chromatography gels chelating with Cu^{2+}, Fe^{3+}, Zn^{2+} and Ca^{2+}

The chelating capacity of the gels with four kinds of metal ions is the predetermined condition for peptide separation by IMAC. Hence, the chelating capacity of affinity chromatography gels with Cu^{2+}, Fe^{3+}, Zn^{2+} or Ca^{2+} was quantitatively determined by atomic absorption spectrometry. As shown in Table II, chelating capacity with Cu^{2+}, Fe^{3+}, Zn^{2+} and Ca^{2+} were 387, 467, 343 and 8.5 μmol per gram of dry gel, respectively. The difference between the groups was significant ($P < 0.05$). The results showed that the chelating capacity per gram of dry gel was highest with Fe^{3+} and lowest with Ca^{2+}. These results agreed data reported by Michele and Thomas, that is, the chelating capacity with Zn^{2+}, Cu^{2+} and Fe^{3+} was 300 ~ 600 μmol per gram of dry gel and that of Ca^{2+} was 8.4 μmol (14).

Table II
Amount of Immobilized Metal Ions on IDA–Sepharose*

	Ca^{2+}	Cu^{2+}	Zn^{2+}	Fe^{3+}
Chelating capacity (μmol/ml wet gel)	1.7 ± 0.3†	77.4 ± 3.2‡	68.6 ± 4.1§	93.5 ± 2.2**
Chelating capacity (μmol/g dry gel)	8.5 ± 1.3†	387.0 ± 7.6‡	343.1 ± 3.5§	467.6 ± 3.6**

*Note: values are the means ± SD ($n = 3$); different symbols in the same row indicate statistically significant differences ($P < 0.05$).

Table III
Binding Amounts of Soybean Peptide to Immobilized IDM–Mn^{+} Adsorbents*

	Ca^{2+}-Sepharose 6B	Cu^{2+}-Sepharose 6B	Zn^{2+}-Sepharose 6B	Fe^{3+}-Sepharose 6B
Sample loaded (mg/g dry gel)	6.67 ± 0.01	6.67 ± 0.01	6.67 ± 0.01	6.67 ± 0.01
Binding capacity (mg/g dry gel)	3.02 ± 0.15†	4.16 ± 0.49‡	3.55 ± 0.18†	4.72 ± 0.29‡
Binding rate (%)	45.23 ± 2.21†	62.44 ± 1.61§	53.27 ± 1.33‡	70.78 ± 2.51**
Binding capacity (g/mol Mn^{+})	354.12 ± 26.32‡	10.75 ± 0.71†	10.35 ± 0.27†	10.09 ± 0.36†

*Values are the means ± SD (n = 3); different symbols in the same row indicate statistically significant differences ($P < 0.05$).

Comparison of soybean peptide binding capacity of prepared chromatography gels

A 20-mg peptide sample, which were prepared in advance, was loaded onto the columns and the quantity of soybean peptide absorbed by the four kinds of IMAC gels was analyzed. The results are shown in Table III. The amounts of peptides bound to Cu^{2+}–IDA–Sepharose 6B, Fe^{3+}–IDA–Sepharose 6B, Zn^{2+}–IDA–Sepharose 6B and Ca^{2+}–IDA–Sepharose 6B were Fe (4.72 mg/g) > Cu (4.16 mg/g) > Zn(3.55 mg/g) > Ca (3.01 mg/g), respectively, with Fe3 + –IDA–Sepharose 6B at the highest binding capacity and Ca2 + –IDA–Sepharose 6B at the lowest. No statistically significant difference was found between Cu^{2+} and Fe^{3+} or between Zn^{2+} and Ca^{2+}. However, the differences between Cu^{2+} and Fe^{3+}, and Zn^{2+} and Ca^{2+} were significant.

Michael and others have reported that horse skeletal muscle myoglobin (HMYO) and sheep skeletalmuscle myoglobin (SMYO), which are rich in aspartic and glutamic acid, exhibited different adsorption efficiency in different IMAC columns (15). Fe^{3+}–IDA–Sepharose 4B had the highest adsorption efficiency, followed by Cu^{2+}–IDA–Sepharose 4B, and Ca^{2+}–IDA–Sepharose 4B had the lowest. The coordination interaction between immobilized metal and protein/peptide depends primarily on the properties of metal ion and protein/peptide surface ligands. When interacting with the same protein, different metal ions display various binding affinities due to the differences of electric charge, ionic radius and electron structures of the metal ions. Cu^{2+}, Ca^{2+} and Zn^{2+} possess identical electric charges, while Fe^{3+} has one more positive charge. In order of decreasing ionic radius, the range is Ca^{2+} (0.99 Å) > Zn^{2+} (0.74 Å) > Cu^{2+} (0.73 Å) > Fe^{3+} (0.645 Å). According to Table III, the binding capacity (mg/g dry gel) of the immobilized metal ions decreased as follows: $Fe^{3+} > Cu^{2+} > Zn^{2+} > Ca^{2+}$. It can be inferred from Table II that the reason Ca^{2+}–IDA–Sepharose binds less protein or peptide is that IDA–Sepharose binds less Ca^{2+}, which may caused by steric hindrance by virtue of a larger ionic radius.

Based on an expression derived from the law of Coulomb (16), the electric work (per mol of charges) W between the metal ions and the charged peptides can be calculated as follows:

$$W = \frac{N_{av} e^2}{4\pi\varepsilon_0\varepsilon_r} \frac{z_p z_m}{r_p + r_m}$$

Here N_{av} is Avogadro's number ($6.022 \times 1023\ mol^{-1}$), e is the electron charge, ε_r is the relative dielectric permittivity of the solvent (78.4) and ε_0 is the dielectric permittivity of the vacuum (8.854×10^{-12} F/m), z_p is the net charge of the peptide, z_m is the binding charge of the metal ion, r_p is the radius of the peptide and r_m is the radius of the metal ion. It could be deduced from this formula that when chelating with the same peptide, W primarily depends on the radius of the metal ions and the charges they carry. In this case, Fe^{3+} possesses the smallest radius and one more charge than other three metal ions, so W between Fe^{3+} and the peptide is also supposed to be the strongest, whereas W of the other three metal ions are in the order of $Cu^{2+} > Zn^{2+} > Ca^{2+}$.

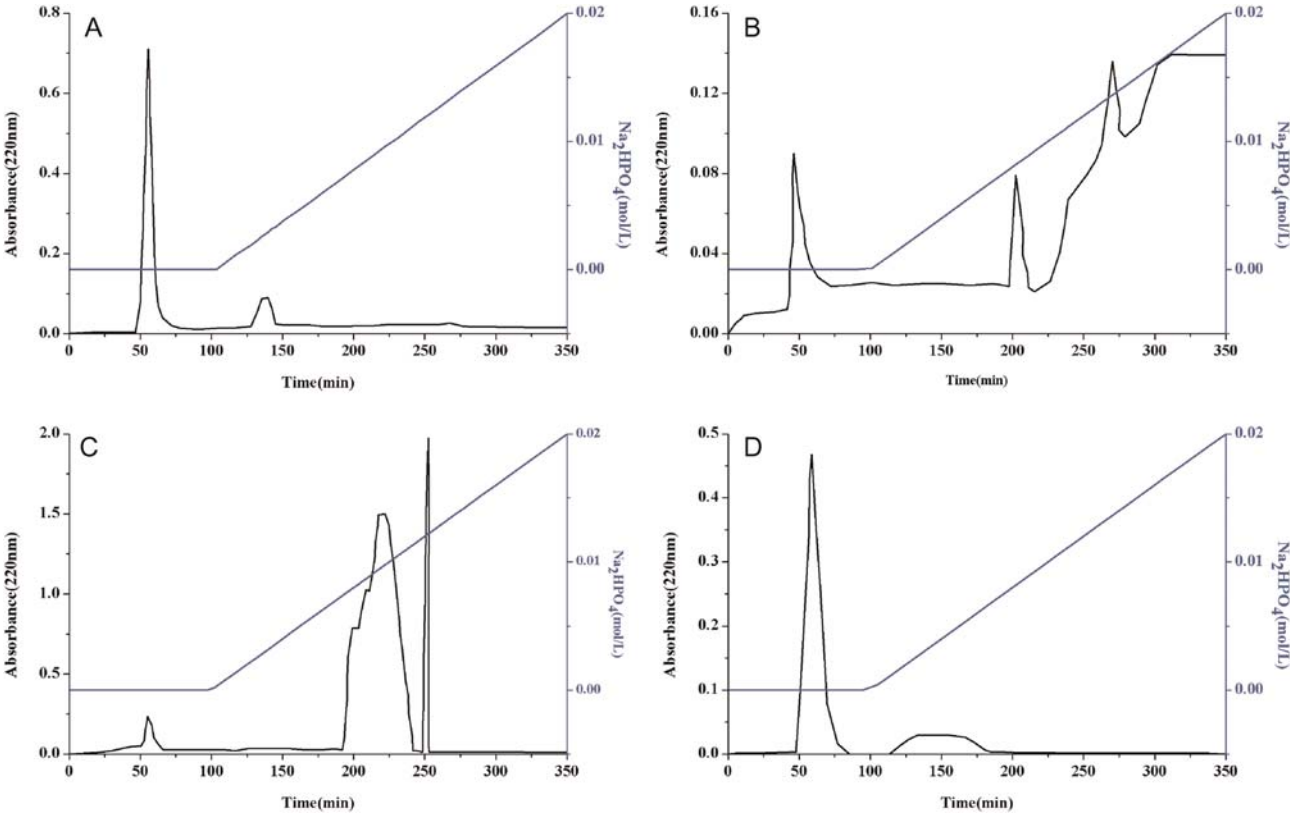

Figure 1. Elution chromatograms of SPH fractions: Ca^{2+}–IDA–Sepharose 6B (A); Fe^{3+}–IDA–Sepharose 6B (B);Cu^{2+}–IDA–Sepharose 6B (C); Zn^{2+}–IDA–Sepharose 6B (D).

However, in terms of binding capacity per mol of metal ion, the affinity effect is in the order of Ca^{2+} (354.12 g/mol Ca^{2+}) > Cu^{2+} (10.75 g/mol Cu^{2+}) > Zn^{2+} (10.35 g/mol Zn^{2+}) > Fe^{3+} (10.09 g/mol Fe^{3+}), and there was a statistically significant differences between Ca^{2+} and the other three metal ions. One simple explanation is that, except for metal ions themselves, peptide structure or sequence also plays an important role in affinity effects between metal ions and peptides. It has been proposed by Michele that certain amino acid residues such as histidine, cysteine and tryptophan might be participating in binding immobilized metal ions (14), but whether these are specifically bound to a certain metal ion or whether different immobilized metal ions induce different changes in peptides are still unknown. We speculate that SPHs might cause structure changes in the Ca^{2+} attachment, such as exposure of amino acids in favor of metal ion binding, causing its high binding capacity (g/mol) to Ca^{2+}. This phenomenon could be explained by the steric mass action (SMA) model, which is usually used to describe protein binding to ion exchangers (17). If ideal behavior is assumed, the equilibrium of the adsorption reaction is given by:

$$K_c \frac{c_P}{q_P} = \left(\frac{c_m}{q_m - \sigma q_p}\right)^{|z_b|}$$

The model has been slightly modified to fit in this experiment. Here, c and q are the liquid and adsorbed phase concentration and the subscripts P and m refer to the peptide and the metal ions, respectively; K_c is the equilibrium constant; z_b is the binding charge and $q_m - \sigma q_P$ means adsorbed metal ions that are available for adsorption, while σ is denoted as the steric factor. That is, not all adsorbed metal ions are available for adsorption because previously adsorbed peptides may shield a number of them. The amounts of Cu^{2+}, Zn^{2+} or Fe^{3+} adsorbed on the column was much larger than Ca^{2+} (Table II); in other words, the density of Cu^{2+}, Zn^{2+} or Fe^{3+} on unit area was higher than Ca^{2+}, which caused more shield effects than Ca^{2+}. In spite of a relatively high binding capacity (g/mol Ca^{2+}), the total amount of Ca^{2+} adsorbed by the column was so small that the binding capacity (mg/g dry gel) of the other three metal ions was still higher.

Based on these theories, Ca^{2+} should be more suitable for chromatographic separation of peptides, provided that more Ca^{2+} can be adsorbed by the column. Comparatively speaking, Fe^{3+} with higher binding capacity (mg/g dry gel) has the advantage.

Separation effects with different IMAC gels

Samples with a concentration of 20 mg/mL were loaded after the four kinds of columns were balanced by 0.1 mol/L NaAC-HAC buffer with NaCl at pH 5.5. Then, disodium hydrogen phosphate, sodium dihydrogen phosphate, sodium glutamate, ammonium chloride, malonic acid and imidazole were employed to elute and the separation effects by the four kinds of prepared IMAC gels were observed. The results showed that acidic sodium dihydrogen phosphate, sodium glutamate and ammonium chloride had poor eluting effects for Cu^{2+}–IDA–Sepharose 6B, Fe^{3+}–IDA–Sepharose 6B and Zn^{2+}–IDA–

Sepharose 6B, whereas alkaline disodium hydrogen phosphate and imidazole had much better eluting effects (data not shown). This phenomenon indicated that soybean peptides were adsorbed under acidic and partial neutral conditions and eluted under alkaline conditions. This is quite different from protein adsorption, which normally takes place in neutral or slightly basic medium (18).

For these reasons, disodium hydrogen phosphate was chosen as the elution buffer to compare the separation effects of the four prepared gels. Elution chromatograms are shown in Figure 1.

As exhibited in the graphs, soybean peptides adsorbed by Ca^{2+}–IDA–Sepharose 6B and Zn^{2+}–IDA–Sepharose 6B (Figures 1A and 1D, respectively) were relatively low, so fractions adsorbed by them needed multiple collections for further study. Cu^{2+} on the IDA–Sepharose 6B (Figure 1C) was easily eluted by a relatively low concentration of alkaline disodium hydrogen phosphate, thus causing the phenomenon of Cu^{2+} leakage. That is, when detected with an online ultraviolet (UV) detector at 220 nm, Cu^{2+} generated a very high absorbance value so that the value caused by the adsorbed fractions could not be effectively read. In this case, we had no choice but to collect the fraction with a high absorbance value as the target peptide adsorbed by Cu^{2+}. The leakage was so severe that after a cycle of elution, the Cu^{2+}-column needed to be regenerated and repacked with Cu^{2+}, whereas the other three columns could stand repeated use. Soybean peptides adsorbed by Fe^{3+}–IDA–Sepharose 6B were relatively high (mg/g dry gel), and there was no leakage phenomenon when it was eluted by a low concentration of alkaline disodium hydrogen phosphate and imidazole. The graphs not only show the differences in separation amounts, but also show that the adsorbed peptides were separated into several fractions. Among all the metal chelated columns, the one with Fe^{3+} exhibited adsorption differences and possessed better separation effects.

Comparison of RP-HPLC profiles of peptides from different gels

It is of great concern that whether adsorbed peptides possess the same properties when separating with different chelated metal ions. For this purpose, HPLC experiments were performed and profiles were obtained of peptides adsorbed by four kinds of metal ions. According to Figure 2, the profiles showed similar patterns. Compared with control loaded peptides, the peptides adsorbed by metal ions had fractions with long retention times in the RP-HPLC profiles. The results illustrated that those peptide fractions displayed strong hydrophobic characters (Figure 2).

As reported previously, metal ions Zn^{2+} and Cu^{2+} have a preference for extra nitrogen-containing amino acids, such as histidine (19–21). In contrast, hard Lewis metal ions, such as Ca^{2+} and Fe^{3+}, prefer oxygen-rich groups of aspartic and glutamic acid or phosphate groups (18, 22–23). Although it is not

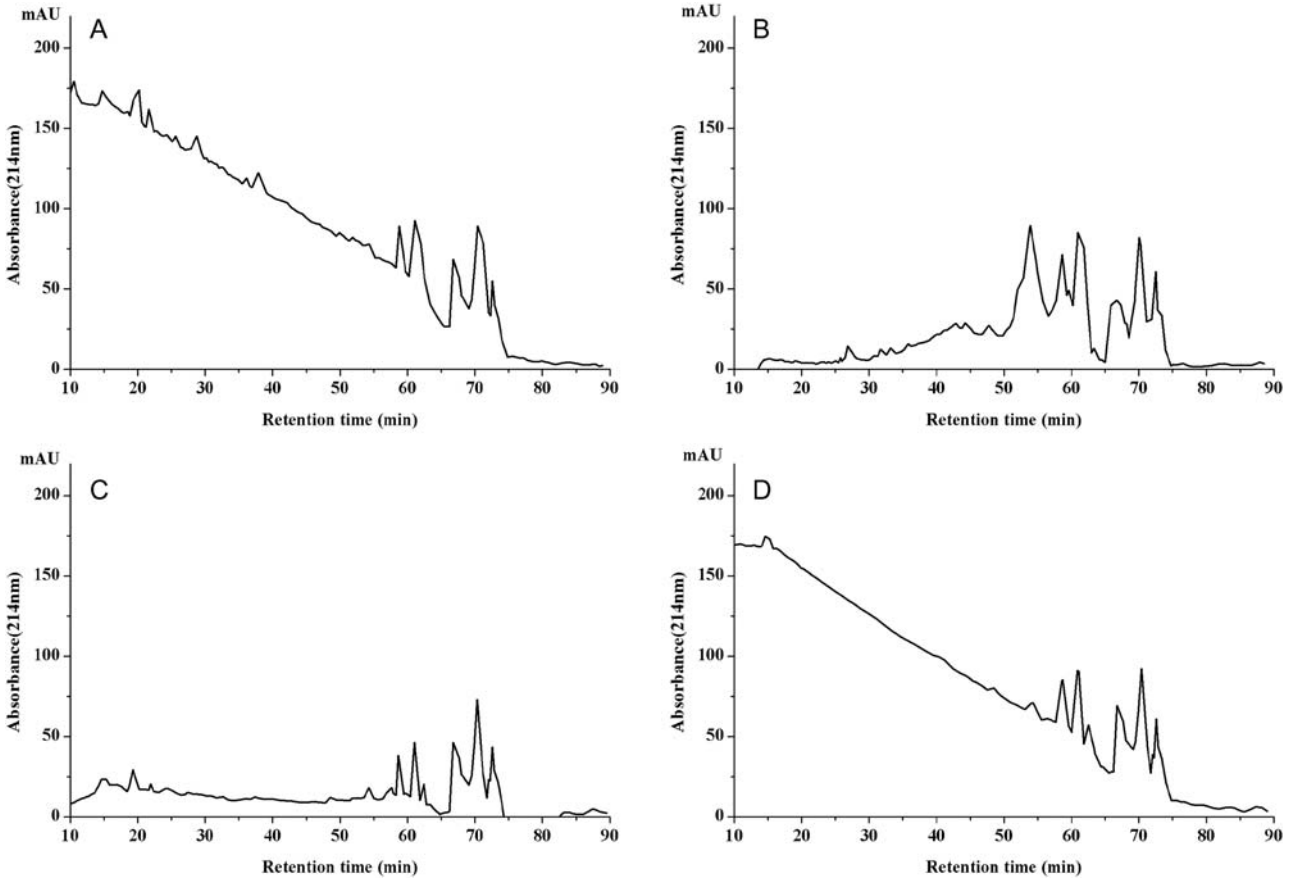

Figure 2. HPLC profiles of peptides adsorbed by Ca^{2+}–IDA–Sepharose 6B (A); Fe^{3+}–IDA–Sepharose 6B (B); Cu^{2+}–IDA–Sepharose 6B (C); Zn^{3+}–IDA–Sepharose 6B (D).

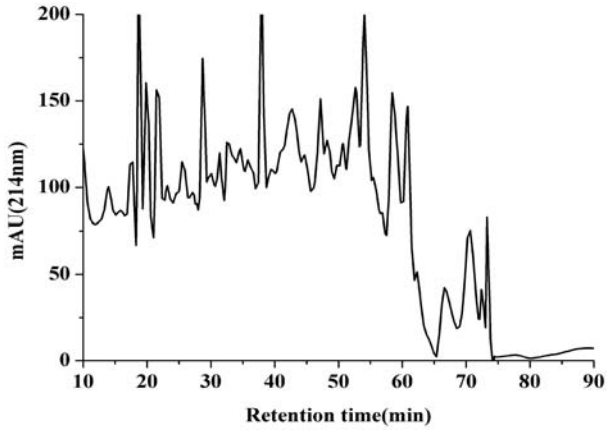

Figure 3. HPLC profile of peptides (10—30 kDa) loaded on the columns.

possible to identify the specific amino acid adsorbed by each metal ion from the HPLC profile, a comparison with the original loaded sample (Figure 3) shows that all of the adsorbed peptides displayed similar hydrophobicity. The results indicated that in addition to the signature amino acid mentioned previously, hydrophobicity is also a crucial factor for interactions between soybean protein hydrolysates and metal ions. In addition, as shown in Figure 2, in spite of intensity differences caused by inconsistent sample concentrations, the peptides adsorbed by the four kinds of metal ions had almost the same separation profile after a retention time of 55 min. This strongly suggests that under the same elution conditions, peptides adsorbed by different metal ions exhibit basically the same hydrophobic property. Furthermore, in separation practice, peptides possessing the same property that can chelate with Ca^{2+}, Cu^{2+} or Zn^{2+} could be prepared using columns with a high capacity of Fe^{3+}.

Conclusions

To obtain the best separation effect, it is of great importance to select the proper immobilized metal ion. In our research, four commonly used immobilized metal ions were evaluated by their chelating capacity on affinity chromatography gels (μmol/g dry gel) and their binding capacity of soybean protein hydrolysates (mg/g dry gel). In terms of binding capacity per mol of metal ion, the affinity effect is in the order of $Ca^{2+} > Cu^{2+} > Zn^{2+} > Fe^{3+}$, and there is a significant difference between Ca^{2+} and the other three metal ions, but no significant difference between the later three ions. However, for chelating capacity on gels and binding capacity of peptides, $Fe^{3+} > Cu^{2+} > Zn^{2+} > Ca^{2+}$. With regard to separation effects, alkaline disodium hydrogen phosphate and imidazole had much better eluting effects, while Cu^{2+} may not be proper to be used as an immobilized metal ion for the SPH separation due to a severe leaking phenomenon.

The HPLC profiles of four metal ions showed great consistency, especially after 55 min, which means peptides adsorbed by the ions were similar in terms of hydrophobicity. The results also indicated that in addition to the existence of certain amino acids, hydrophobicity might favor the metal ions and interactions of adsorbed peptides . In separation practice, when calcium-chelating peptides are hard to collect because of the poor chelating capacity of Ca^{2+} on the gels, Fe^{3+} with high chelating capacity can be utilized for sample preparation.

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